The diagnostic value of microsatellite LOH analysis and the prognostic relevance of angiogenic gene expression in urinary bladder cancer

Doctoral theses

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Budapest, 2008
1. INTRODUCTION

Approximately 330,000 new cases of bladder cancer are diagnosed each year, and accounts for approximately 178,000 cancer deaths per year worldwide, 809 in Hungary.

Currently the histological finding is the only factor that determines the therapy and patients prognosis. Based on histological examination, bladder cancer can be divided into superficial and muscle invasive cancer. Superficial bladder cancer involves the bladder wall no deeper than the subepithelial tissue and does not reach the detrusor muscle. For patients with non-invasive bladder cancer, the five-year survival rate is over 90%. The rate drops below 40%, if the cancer spreads to the muscular layer of the urinary bladder. This emphasizes that early diagnosis is crucial to survival.

Bladder cancer is associated with cigarette smoking and a number of occupational exposures. Chemicals found in some dyes, paints, solvents, leather dust, inks, combustion products, rubbers, and textiles can increase the risk of bladder cancer. Increased risk of bladder cancer has also been observed among hairdressers, machinists, painters, printers, truck drivers, and those who work with the drugs used in chemotherapy. There is clearly a need for appropriate screening methods to detect bladder cancer in these high-risk groups.

Correct staging is important because the treatment requirements of Ta or T1 high-grade tumor and T2 tumor are completely different. There is a significant risk of understaging after the initial resection, since the surgical resection line often does not reach the layer of tumor invasion or because the biopsy is heat damaged. In such cases a second resection is required which may lead to a considerable delay in starting appropriate therapy.

A potential marker for tumor invasion may help to confirm or modulate unclear histological findings resulting in considerable benefit to patients’ management.

Beside early diagnosis and accurate histological assessment predicting disease prognosis is another important issue in bladder cancer. After initial treatment of superficial bladder cancer 70% of patients develop recurrent tumors and the overall risk of muscle-invasive cancer is 5-15%. High-risk groups for tumor recurrence and stage-progression could benefit from an early, more aggressive therapy. In muscle invasive bladder cancer the most important question is if the tumor is able to spread to other organs. The main risk in muscle invasive bladder cancer is metastasis. While the 5-year survival rate in non-metastatic invasive bladder carcinoma is 39%; it is only 12% in metastatic disease. Therefore the early identification of high-risk cases could translate in an increase in curing rate.

Multiple genetic changes are involved in the evolution and progression of human cancer. These changes can be detected in the urine by analysis of the cells exfoliated from the bladder wall, enabling us to detect bladder cancer. However, the presence of normal DNA derived from non-cancerous cells might interfere with the analysis. The ratio between the normal and cancerous epithel cells in the urine sediment is probably related to the tumor size. Therefore, analysis of the urine sediment provides a less sensitive result in noninvasive cases. On the other hand, the free DNA level in body fluids is elevated in cancer patients. The urine supernatant is suggested to contain cell-free DNA that mainly originates from the tumor.

Microsatellites are short, polymorphic, tandem repeat segments dispersed throughout the human genome. Microsatellite markers are considered to be effective in detecting genetic alterations, such as microsatellite instability and loss of heterozygosity (LOH) in human cancer.
Current studies focused on the detection of bladder cancer and not on the discrimination of tumors with different stages.

Angiogenesis, the formation of new vessels from the existing ones, is essential for the growth and progression of solid tumors. This process begins when the tumor colony expands to a size where simple diffusion of oxygen and nutrients are insufficient. The subsequent hypoxia results in a switch in the net balance between activators and inhibitors of angiogenesis towards the activators (angiogenic switch), resulting in blood vessel growth. Although vascular endothelial growth factor (VEGF), angiopoietins (Ang-1 and Ang-2) and their receptor Tie2 do play a key function in the angiogenic process, their role in the formation of recurrence and progression is still unknown.

II. OBJECTIVES

In the first part of this study we focused on the detection of genetic alterations in urine.

We sought correlation:
- between presence of genetic alterations and bladder cancer (non-invasive tumor detection)
- between deletion of specific chromosomal regions and tumor stage and grade (to support histological findings in uncertain cases)

In order to do this we performed:
- microsatellite loss of heterozygosity (LOH) analysis
  - using 12 polymorphic microsatellite markers to test the feasibility in our laboratory conditions
  - using a genom-wide panel of 400 microsatellite markers (whole genom microsatellite analysis) to find the chromosomal regions their deletion correlates with tumor-stage and grade
- fluorescent in situ hybridization (FISH) using UroVysion Kit

In the second part of this study we analyzed the prognostic value of angiogenic factors (VEGF, Ang-1, Ang-2) and their receptor Tie2 in urinary bladder cancer.
III. MATERIALS AND METHODS

III. 1. Microsatellite LOH analysis (12 markers)

Matched blood, urine and tumor samples were collected from 44 patients (13 female, 31 male; the average age was 74 years) who underwent surgical resection of bladder cancer at the Department of Urology. The diagnosis was confirmed and graded after surgical resection by histological examination. Blood and urine samples were also collected from 20 control patients with non-malignant urinary diseases and 16 healthy individuals without any previous serious urological anamnesis.

DNA was extracted from blood, tumor and urine samples (supernatant and sediment). Polymerase chain reaction (PCR) was performed using twelve fluorescently labeled microsatellite-specific primers. The PCR products were separated using capillary electrophoresis. Cases where the peak height ratio of normal (blood) sample differed more than 50% from peak height ratio of the analyzed sample (urine or tumor) were considered as LOH.

III. 2. Whole-genom microsatellite LOH analysis (400 markers)

We analyzed blood and tumor samples of 17 patients who underwent transurethral resection in the Department of Urology of the University Hospital Essen. DNA extraction was performed followed by PCR using 400 microsatellite markers mapped on all chromosomes, providing a resolution of 10 cM. The PCR product separation and data evaluation was performed as described above.

III. 3 UroVysion FISH analysis

Urine samples of 43 bladder cancer patients and 12 controls were analyzed by a commercially available fluorescent in situ hybridization kit. The UroVysion kit comprises a mix of probes for the detection of the 9p21 locus as well as the peri-centromeric region of chromosome 3, 7 and 17. The urine sample preparation and the hybridization were performed according to the manufacturers’ instructions. Cytogenetic abnormalities fulfilling the positivity criteria of the UroVysion Bladder Cancer Recurrence Kit were determined according to instructions: the sample was considered positive if no 9p21 signals were found in at least 12 cells or if at least two of CEP3, CEP7 and CEP17 showed gain in at least 4 cells. At least 25 abnormal cells were counted.

III. 4. Genexpression analysis of angiogenic factors

Tumor samples of 113 bladder cancer patients and normal bladder epithelium of 5 non-cancer patients were collected. After RNA isolation reverse transcription was performed. The gene expression levels of VEGF, Ang-1, Ang-2 and Tie2 were determined by quantitative real-time PCR (Q-RT-PCR) using the $\Delta \Delta C_T$ method.
IV. RESULTS

IV. 1. Microsatellite LOH analysis (12 markers)

Microsatellite alterations were detected in urine sediment and supernatant in 86% of the cancer cases. Urine sediment alone had a sensitivity of 68%, while urine supernatant alone indicated aberrations in 80% of the tumors. In superficial (Ta/T1) cases, a considerable difference in sensitivity (84% vs. 67%) was found between the two fractions in favour of urine supernatant. The specificity of microsatellite analysis was higher in urine sediment as in urine supernatant (95% vs. 80%).

IV. 2. Whole-genom microsatellite LOH analysis (400 markers)

Analyzing 13 Ta and 4 T1 bladder cancer specimens and matched blood samples, we identified chromosomal regions frequently deleted in early stages of bladder cancer. The uneven distribution of chromosomal deletions in superficial cases suggests that alterations of chromosome 9 play a causal role in development of this disease. Among the 16 most common deleted microsatellite regions, 15 localized on chromosome 9 and one on chromosome 11 (D11S905). We detected significantly higher number of deletions in T1 tumors than in Ta tumors (p=0.05). According with this observation we identified 9 microsatellite regions (D4S403, D5S422, D5S436, D5S641, D9S164, D9S1682, D11S338, D11S904 D17S799) they are frequently deleted in T1 tumors but not in Ta tumors. Analysis of these regions may discriminate between Ta and T1 tumors.

Furthermore we defined a microsatellite marker combination that was able to detect all the 17 bladder cancer cases.

IV. 3 UroVysion FISH analysis

Positivity criteria of UroVysion test were met in 34 of 43 bladder cancer cases. Negative results were obtained in 16 cases from which 5 proved to be superficial carcinoma. Based on these the sensitivity was 87%. None of the control urines proved positive results, thus the specificity was 100%.

IV. 4. Gene expression analysis of angiogenic factors

In tissues of non-invasive bladder tumors Ang-1 expression was significantly lower (p<0.001) while VEGF expression was significantly higher (p=0.031) than in normal bladder tissue. In contrast Tie2 and Ang-2 abundance in tumor did not differ significantly from that in normal bladder tissue. Multivariate analysis identified Ang-2 as a strong and independent predictor of tumor recurrence (hazard ratio [HR] =10.18, 95% CI 2.69-38.49, p<0.001) and Tie2 expression as an independent favorable prognostic factor for both metastasis (HR=0.31, 95%CI 0.11-0.89, p=0.029) and disease-specific survival (HR=0.25, 95% CI 0.10-0.62, p=0.003).
V. DISCUSSION

The present work tries to answer some current diagnostic and prognostic questions of urinary bladder cancer using new molecular methods.

In the diagnostic applications, we focused on detection of DNA alterations in human urine samples. We installed the UroVysion FISH method and microsatellite deletion analysis and defined their specificity and sensitivity in our laboratory conditions. The analysis of cell-free DNA of urine supernatant provided higher sensitivity as urine sediment. Performing a genom wide screening by a set of 400 microsatellite markers (mapped on all chromosomes). We were able to reduce the minimal number of microsatellite primers to 4 with a sensitivity of 100%. Furthermore we identified chromosomal regions deleted only in muscle-invasive bladder tumors. The analysis of these regions would have an importance in cases with unclear results of histological examination.

Based on their molecular characteristics, future behavior of tumors become more and more predictive. The prognostic value of angiogenesis was confirmed by both morphologic and molecular biologic evidence.

We identified tissue mRNA expressions and serum protein concentrations of five angiogenic factors critically involved in normal and pathological angiogenesis. To explore these markers clinical relevance, their expression data was compared with a long follow-up period.

We found a characteristic “angiogenic switch” in gene expression pattern with strong down-regulation of Ang-1 and concurrent up-regulation of Ang-2 and VEGF expression in bladder tumor stage pTa, a superficial non-invasive tumor stage. This shift is probably a main driving force of vascular destabilization and initiation of angiogenesis in bladder cancer. Remarkably, this switch is less pronounced in later stages of bladder cancer.

We identified Ang-2 and VEGF as independent predictors of disease recurrence and demonstrated the predictive potential of Tie2 expression for bladder cancer metastasis and disease-specific survival.

The detailed results above show that the expression of angiogenic factors does have a significant impact on patients’ survival in bladder cancer. This indicates the need for new antiangiogenic therapy modalities in this disease. The advances on this field lay claim to markers to measure the biological effects of targeted agents and to identify patients who most likely benefit from the treatment.
VI. CONCLUSIONS

1. Microsatellite analysis and UroVysion FISH analysis of the urine are efficient and noninvasive molecular methods to detect bladder cancer.

2. Genom-wide microsatellite LOH analysis demonstrated the existence of chromosomal regions their deletions do correlate with bladder tumor progression. Further analysis of these regions may reveal tumor suppressor genes critically involved in cancer progression and could contribute to a more accurate tumor staging.

3. The gene expression pattern of angiogenic cytokines we found in different stages suggests the strongest proangiogenic signal in early stages of bladder cancer.

4. The factors playing a leading role in tumor induced angiogenesis may help to predict disease prognosis.
   - High Ang-2 and VEGF expression are significant risk factors for recurrence in superficial bladder cancer.
   - High VEGF and low Tie2 are unfavorable predictors of tumor metastasis in muscle-invasive bladder cancer.

VII. PUBLICATION RECORD

VII. 1. Publications in the subject of the dissertation


   IF: 1.597

   IF: 1.272

   IF: 1.272

   IF: 6.250

VII. 2. Publications in different subject

   IF: 3.525


\section*{VII. 3. Abstracts}


4. Firneisz G. Dudás J, Szarvas T, Sári E, Ramadori G, Kovalszky I. Different transforming growth factor-B1 induces extracellular matrix production in rat hepatic stellate cells versus hepatic myofibroblasts, but decorin inhibits both. \textit{Z. Gastroenterol}, 44 (5) \textbf{IF: 0.800}

\section*{VII. 4. Presentations, posters}


11. Firneisz G., Dudás J., **Szarvas T.**, Lengyel G., Fehér J., Ramadori G., Koválszky I. Different transforming growth factor-B1 signaling in rat hepatic stellate cells compared to hepatic myofibroblasts (poszter) 14th United European Gastroenterology Week, Berlin, Germany, 2006. október